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# Discrimination of COVID-19 from inflammation-induced cytokine storm syndromes by disease-related blood biomarkers

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## Abstract

**Objectives:** Infection with the novel coronavirus SARS-CoV-2 triggers severe illness with high mortality in a subgroup of patients. Such critical course of coronavirus disease (COVID)-19 is thought to associate with cytokine storm as in macrophage activation syndrome (MAS) or secondary hemophagocytic lymphohistiocytosis (sHLH), although these specific data are still lacking. In this study we aimed to directly address the question, whether immune activation in COVID-19 does indeed mimic conditions as in these classical cytokine storm syndromes.

**Methods:** We quantified levels of 22 biomarkers in serum samples of COVID-19 (n=30, n=83 longitudinal samples in total), sHLH/MAS patients (n=50) as well as healthy controls (n=9) using bead array assay as well as single-marker ELISA and correlated results with disease outcome.

**Results:** In sHLH/MAS we observed dramatic activation of the interleukin(IL)-18-interferon (IFN)-γ axis, while increased serum levels of IL-1 receptor antagonist (IL-1Ra), intracellular adhesion molecule 1 (ICAM-1) and IL-8, as well as strongly reduced levels of soluble Fas ligand (sFasL) in course of SARS-CoV-2 infection discriminate immune dysregulation in critical COVID-19 from the investigated well-recognized cytokine storm conditions.

**Conclusions:** Serum biomarker profiles clearly separate COVID-19 from MAS or sHLH, which questions the significance of systemic hyperinflammation following SARS-CoV-2 infection as well as the efficacy of drugs targeting key molecules and pathways specifically associated with systemic cytokine storm conditions in the treatment of COVID-19.


**Key words:** COVID-19, cytokine storm, macrophage activation syndrome, biomarkers

# Introduction

The novel coronavirus SARS-CoV-2 is infecting ever increasing numbers of people around the globe. While the infection results in mild to moderate symptoms in most individuals, it triggers a severe illness with high mortality in a subgroup of patients.

Already early in the pandemic, severe (fatal) course of COVID-19 was proposed to correlate with hyperinflammation as seen in classic cytokine storm syndromes (1). Those include secondary hemophagocytic lymphohistiocytosis (sHLH) which may occur, for example, in the context of infection, malignancy, metabolic disease, trauma or rheumatic disease (in the latter case referred to as macrophage activation syndrome, MAS). MAS is particularly associated with adult-onset Still's disease (AOSD) and systemic juvenile idiopathic arthritis (sJIA) in children, but is also seen in Kawasaki Disease and other rheumatic conditions. Current data suggest a strong clinical and immunophenotypic overlap between sHLH and MAS (2).

Key molecules or pathways that drive HLH/MAS such as IL-1β, IL-6, IL-18, IFN-γ or JAK/STAT can be targeted by state-of-the-art therapies, and ever since the proposal regarding an overlap of (critical) COVID-19 with classical cytokine storm conditions has been put forward (1, 3), those have been considered as therapeutic targets in COVID-19 or are already studied in respective clinical trials (NCT04372186, NCT04317092, NCT04324021, NCT04338958). Yet, at the same time, studies addressing the relevance of cytokine storm conditions in COVID-19 are frequently limited to discussions on IL-6(4) and draw conclusions based on comparisons with many different critical clinical conditions or even healthy controls. However, to draw such conclusions we believe it is necessary to investigate severe immunological scenarios, that are classified as "cytokine storm conditions" by respective clinical and laboratory criteria. Therefore, in this study we set out to directly compared cytokine signatures in sHLH and MAS with those in COVID-19 and describe serum biomarkers which clearly separate the different entities.

# Methods

### Study subjects and samples

Serum samples (n=83) from COVID-19 patients (n=30) were collected at the Department of Gastroenterology, Hepatology, Endocrinology and Clinical Infectiology, University Hospital Muenster, Germany (03-05/2020). Samples were collected ranging from hospital admission throughout the disease course. All COVID-19 patients' samples were collected during the first COVID-19 wave in Germany and none of the enrolled COVID-19 patients received

immunosuppressive or biologic therapies or (experimental) anti-viral treatment. However, patients received anti-infective drugs in cases of bacterial or fungal superinfection. Disease severity was defined as critical (presence of acute respiratory distress syndrome [ARDS] and/or deceased), severe (requiring oxygen supplementation) or moderate (neither ARDS was present nor oxygen supplementation required). ARDS was diagnosed according to the Berlin definition (bilateral opacities on chest radiograph, exclusion of other causes of respiratory failure) (5). COVID-19 patients were categorized according to their respective worst condition over the course of hospitalization.

Included adult sHLH (n=20) and AOSD-MAS serum samples (n=17) were collected in course of previous studies (6-8). Serum samples of pediatric/adolescent sHLH (n=4) and MAS patients (n=9) and healthy controls (n=9) were collected at University Children's Hospital Muenster, Germany. Secondary HLH and MAS samples were collected during active disease and disease classification is further detailed in the **supplementary methods section** as well as **supplementary table S1**.

All study subjects or care givers provided written informed consent. The study was approved by the already reported(6-8) as well as local (University Hospital Muenster: 2020-210-s-S, 2015-670-f-S) ethical committees.

**Quantification of serum markers**

Reagents for multiplexed quantification of IL-1β, IL-1Ra, IL-4, IL-6, IL-8, IL-10, IL-18, TNFα, IFNα, IFNβ, IFN-γ, MCP2 (CCL8), MCP3 (CCL7), CXCL9, CXCL10, MCSF, LRG1, sFasL, ICAM-1, VCAM-1 and Galectin-3 were purchased from R&D Systems (Minneapolis, OH, USA). Reagents and sera were prepared according to the manufacturer's instructions (R&D Systems). Data acquisition and analysis was performed on a MAGPIX instrument (Merck Millipore, Darmstadt, Germany) using xPONENT v4.2 software (Luminex). Concentrations of S100A12 in patients' sera were quantified by sandwich ELISA using in-house monoclonal antibodies.

**Data analysis**

Serum marker data were analyzed for unsupervised clustering using correlation distance and ward.D linkage by the pheatmap R package and Rstudio (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA http://www.rstudio.com/). Principle component analyses were performed using the ggfortify and autoplot R packages and Rstudio.

Multiple spearman rank correlation analyses of serum analytes were performed and plotted using the corrplot R package and Rstudio or Graphpad Prism software (Version 8.0 for Mac OS X, Graphpad Software, La Jolla, CA, USA).

Data of individual serum markers were analyzed for normality distribution by D'Agostino & Pearson normality test using Graphpad Prism software. The large majority of data did not pass this test and were therefore subjected to non-parametric multi-comparison analyses by Kruskal Wallis followed by Dunn's multiple comparison test (Graphpad Prism v8.0). Receiver operating characteristic (ROC) curve analyses were performed using Graphpad Prism software.

# Results

**Serum marker profiling of COVID-19 compared to classic cytokine storm syndromes**

In our cohort of COVID-19 patients (n=30, **table 1**) hospitalized during the first wave of SARS-COV-2 infections, seventeen patients had critical disease, of whom 7 died. Six patients presented with severe disease and seven were classified as moderate disease. Unsupervised hierarchical clustering (**Figure 1A**) and principal component analyses (PCA, **Figure 1B-D**) of early (1$^{st}$ blood sampling following hospitalization) serum marker profiles of COVID-19 compared to sHLH/MAS (n=50) patients as well as healthy controls (n=9, **Tables 1 and S1**) revealed a distinct grouping of patients. PCA discriminated critical COVID-19 patients from those with severe and moderate disease, which rather clustered with healthy controls (**Figure 1B**). Patients with sHLH/MAS clustered separately from those with COVID-19 (**Figures 1C, D**), particularly when comparing sHLH and MAS *versus* COVID-19 individually (**Figures 1A** and **1D**).

In COVID-19 with critical course, the majority of assessed biomarker levels, were elevated in similar range as in sHLH and MAS (**Figure S1A**). No differences in serum levels of IFN-α, IFN-β and MCP2 were noted (**Figure S1B**). However, we observed six serum markers to separate sHLH/MAS from critical and/or severe COVID-19 (**Figure 1E**). Levels of IL-18 and IFN-γ were markedly elevated in sHLH and MAS, while the IL-18/CXCL9 ratio discriminated only MAS from critical COVID-19 (**Figure S1C**). Serum concentrations of IL-8 and IL-1Ra were significantly increased in critical COVID-19 compared to MAS or sHLH, respectively. Further, serum levels of soluble ICAM-1 were increased in critical disease COVID-19 compared to both sHLH and MAS. In contrast to these elevated markers, serum levels of sFasL were markedly

decreased in COVID-19 compared to both sHLH and MAS (**Figure 1E**). In contrast to the included COVID-19 patients, some sHLH and MAS patients received immunosuppressive medication (**Table S1**). However, when removing those samples from the data set, this has only little effect on the previously recorded significances between markers reported in **figure 1E** (**Figure S2**).

**Selected serum biomarkers separate critical COVID-19 from classic cytokine storm syndromes irrespective of disease severity**

Over the course of the disease inflammatory biomarker levels in sera of COVID-19 patients vary with respect to sampling time point from first manifestation of symptoms (**Figure S3**). When comparing serum marker levels in the first available sample following hospitalization (days from first symptoms, median (IQR):12.5 (11-21)) with the respective last (31 (21-36)), we noted biomarker concentrations in some critical disease COVID-19 patients to have escalated in the disease course, while in others levels rather approached those of healthy controls (**Figure S4**). However, none of these changes reached significance level. When analyzing samples collected early *versus* late in the disease course, the underlying serum marker signatures still clearly distinguished sHLH from critical COVID-19, regardless of the time point of sample collection (**Figure S5**). ROC analyses of specifically IL-18, IFN-γ, sFasL and ICAM-1 serum levels collected at different time points during critical COVID-19 course revealed almost identical performance in separating critical COVID-19 from sHLH and MAS (**Figure 1F**; **Table S2**). In contrast, IL-1Ra and IL-8 serum levels quantified in samples collected late in the course of critical COVID-19 revealed less power in separating critical disease COVID-19 patients from either sHLH (IL-1Ra) or MAS (IL-8), compared to respective serum concentrations in samples collected early in disease course (**Figure 1F**; **Table S2**). When testing the identified parameters for their power in differentiating sHLH or MAS *versus* critical COVID-19 patients' samples collected within defined time frames post onset of first symptoms, this confirms a universal strong differentiation of critical disease COVID-19 from both sHLH and MAS by IL-18, IFN-γ, sFasL and ICAM-1 serum levels (**Figure S6**). Of all tested markers, IFNγ serum levels could best separate sHLH and MAS from critical COVID-19 (**Figure 1F**; **Table S2**).

**Dysregulation of the IL-18-IFN-γ axis in classic cytokine storm syndromes compared to COVID-19**

IL-18 and IFN-γ have a central role in viral defense(9), but also in the pathogenesis of hyperinflammation as in sHLH/MAS(2). Importantly, our serum biomarker analyses revealed a pronounced differential expression of these cytokines in SARS-COV2-induced inflammation compared to sHLH/MAS. In multiple correlation analyses in sHLH/MAS we noted a prevalence of positive associations of both IL-18 and IFN-γ with almost all quantified serum markers (**Figure 2A**), which we did not observe in critical COVID-19 (**Figure 2B**) and this was similarly true for many other blood biomarkers. When further analyzing associations of IL-18 with levels of IFN-γ or the IFN-γ signaling surrogates CXCL9 and CXCL10 as well as serum ferritin and thrombocyte counts, as previously established to confirm a role of IFN-γ in MAS pathogenesis(10), we noted poor correlation of these parameters in critical COVID-19 (**Figure 2A, B**). Although serum ferritin levels and blood thrombocyte counts did not differ significantly between critical COVID-19 and sHLH/MAS (**Figure S7**), correlation of those with other investigated parameters was strikingly different in sHLH/MAS compared to COVID-19 (**Figure 2C, D**).

## Discussion

The initial proposal of a cytokine storm as a relevant element of (critical) COVID-19 pathogenesis(1) intrigued physicians and researchers, particularly in the field of rheumatology, as here such conditions are seen and investigated on a regular basis(3). However, while the scientific discussion on relevance and impact of cytokine storm following SARS-COV-2 infection is still ongoing(11), to our knowledge there are yet no data which explicitly compare the immunology in COVID-19 with classical, inflammation-induced cytokine storm conditions as defined by respective clinical and laboratory criteria. Therefore, in this study we analyzed serum biomarker signatures of COVID-19 compared to sHLH and MAS as classic cytokine storm syndromes and found the IL-18-IFN-γ axis as well as levels of sFasL and ICAM-1 to clearly differentiate those from SARS-CoV-2-induced immune dysregulation.

In our patient cohort quantified inflammatory serum markers in COVID-19 increased with disease severity and could indicate disease outcome already early in the course, which supports previous data(12). In COVID-19 with critical course, the majority of assessed biomarker levels, including IL-6, were elevated in similar range as in sHLH and MAS. Importantly, none of the enrolled COVID-19 patients received immunosuppressive or biologic therapies or (experimental) anti-viral treatment which may confound the obtained results.

In contrast to many quantified parameters, particularly the IFN-γ axis, including IL-18 as IFN-γ-inducing factor(9) as well as IFN-γ itself, appears deregulated in sHLH and MAS, which echoes previous data(10). While reduced IFN-γ expression in COVID-19 has already been reported earlier(13), we now show that this as well as IL-18 significantly tells apart COVID-19 from hyperferritinemic cytokine storm conditions.

While the IL-18-IFN-γ axis appears dramatically deregulated in sHLH and MAS and corresponding serum levels of these cytokines can be found in substantially increased concentration ranges compared to COVID-19, this is similarly true for serum concentrations of sFasL. However, here the differences arise from strongly decreased levels in critical COVID-19 compared to both healthy controls and sHLH or MAS patients. Decreasing sFasL-levels according to COVID-19 disease activity which phenocopy our data have been reported very recently(14) and may indicate a selective SARS-CoV-2-induced immunosuppressive effect rather than general overactivation and hyperinflammation(15). Further, these data could point to an evasive strategy from apoptosis as previously reported for HIV on the level of FasL expression(16).

In contrast to IL-18, IFN-γ and sFasL, serum concentrations of ICAM-1 were significantly elevated compared to both sHLH and MAS. Increased soluble ICAM-1 in COVID-19 patients' sera has already been reported earlier and is suggested to indicate excessive endothelial activation and barrier dysfunction(17). Within our data set we observed similar changes in soluble VCAM-1, albeit those remained below significance level.

Similar to ICAM-1, IL-8 and IL-1Ra serum levels were significantly increased in particularly critical COVID-19 but not in MAS or sHLH, respectively. Elevated serum levels of these markers can indicate general inflammatory activity but with respect to the specific clinical presentation of critical disease COVID-19 patients, increased IL-8 serum concentrations may indeed reflect ARDS pathology. In ARDS patients, IL-8 has been shown to enable both neutrophil influx and survival in lung tissue(18). Correspondingly, therapeutic efficacy of IL-8-blockade is currently tested in COVID-19 (NCT04347226).

While our analyses suggest particular inflammatory axes to contrast COVID-19 from inflammation or infection-induced cytokine storm as in MAS or sHLH, we are well aware of three limitations of our study: First, it is descriptive and limited to a rather small number of patients. Second, even though we significantly extend beyond IL-6(4), our serum marker panel still comprises comparably few analytes, but covers those with reported relevance in classic cytokine

storm conditions(2). Third, we enforce a comparison between serum marker signatures in clinical conditions with a predominant lung (COVID-19) *versus* systemic pathology (sHLH/MAS).

Yet, despite these limitations we believe our data to provide important insights on the proposed overlap between SARS-CoV-2 induced immune dysregulation and classical cytokine storm conditions(3) and to rather question the significance of systemic hyperinflammation in COVID-19(19). Our analyses may further raise doubt regarding the efficacy of clinical trials targeting key molecules and pathways associated with sHLH and/or MAS in the treatment of COVID-19. Therapeutic blockade of IFN-γ, which appears as promising therapeutic option in treating HLH(20) and potentially also MAS(21) may be less effective in COVID-19 (NCT04324021) as the overall activation of the IL-18-IFN- γ axis seems far less pronounced in context of SARS-CoV2 infection. In contrast to IL-18 and IFN-γ, IL-1Ra levels in COVID-19 are substantially elevated. This observation may point to a limited utility of therapeutic IL-1 blockade in patients with COVID-19(22, 23) since high endogenous levels of IL-1Ra have been reported to indicate rather poor response to drugs neutralizing IL-1β or IL-1-signaling(24). However, elevated circulating concentrations of IL-1Ra usually reflect an IL-1 signature, such as the correct timing of IL-1 blockade in COVID-19 may be critical and likely complicates the interpretation of present data (22). Thus, early intervention upon acute hyperinflammatory respiratory failure can have a therapeutic effect (25-27). Further, albeit at a different level compared to sHLH/MAS, the IL-18-IFN-γ axis is certainly active in critical COVID-19 and targeting this and IL-1 simultaneously may constitute a rescue treatment for extremely ill patients (28). A corresponding RCT is ongoing. Indeed, our data may further support the use of combined medications directed against different targets, medications with broader immunoregulatory effects, such as glucocorticoids/ dexamethasone(29), or suggest strategies to bypass low sFasL expression or block IL-8 signaling in treating COVID-19 (NCT04347226).

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**Tabel 1.** Patient characteristics

| **Characteristics** | | **COVID-19 (n=30)** | | | | **sHLH/MAS (sHLH, n=22; MAS, n=28)** | **HC (n=9)** |
|---|---|---|---|---|---|---|---|
| | | | Critical disease (CD), n=17 | Severe disease (SD), n=6 | Moderate Disease (MD), n=7 | | |
| Sex, No. (%) | Male | 28 (93) | 16 (94) | 6 (100) | 6 (86) | 24 (48) | 4 (44) |
| | Female | 2 (7) | 1 (6) | 0 (0) | 1 (14) | 26 (52) | 5 (55) |
| Age, median (range) | | 57 (30-81) | 60 (49-76) | 53 (49-73) | 54 (30-81) | 48 (1.5-86.5) | 28 (7-55) |
| BMI, median (IQR) | | 25 (23-29) | 27 (24.5-30.5) | 23 (22.8-25.3) | 23 (22-26) | n.d. | n.d. |
| Medical history, No. (%) | | | | | | | |
| | Cardiovascular insufficiency | 4 (13) | 3 (18) | 0 (0) | 1 (14) | 0 (0) | 0 (0) |
| | Respiratory insufficiency | 1 (3) | 1 (6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | COPD | 1 (3) | 1 (6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Kidney insufficiency | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

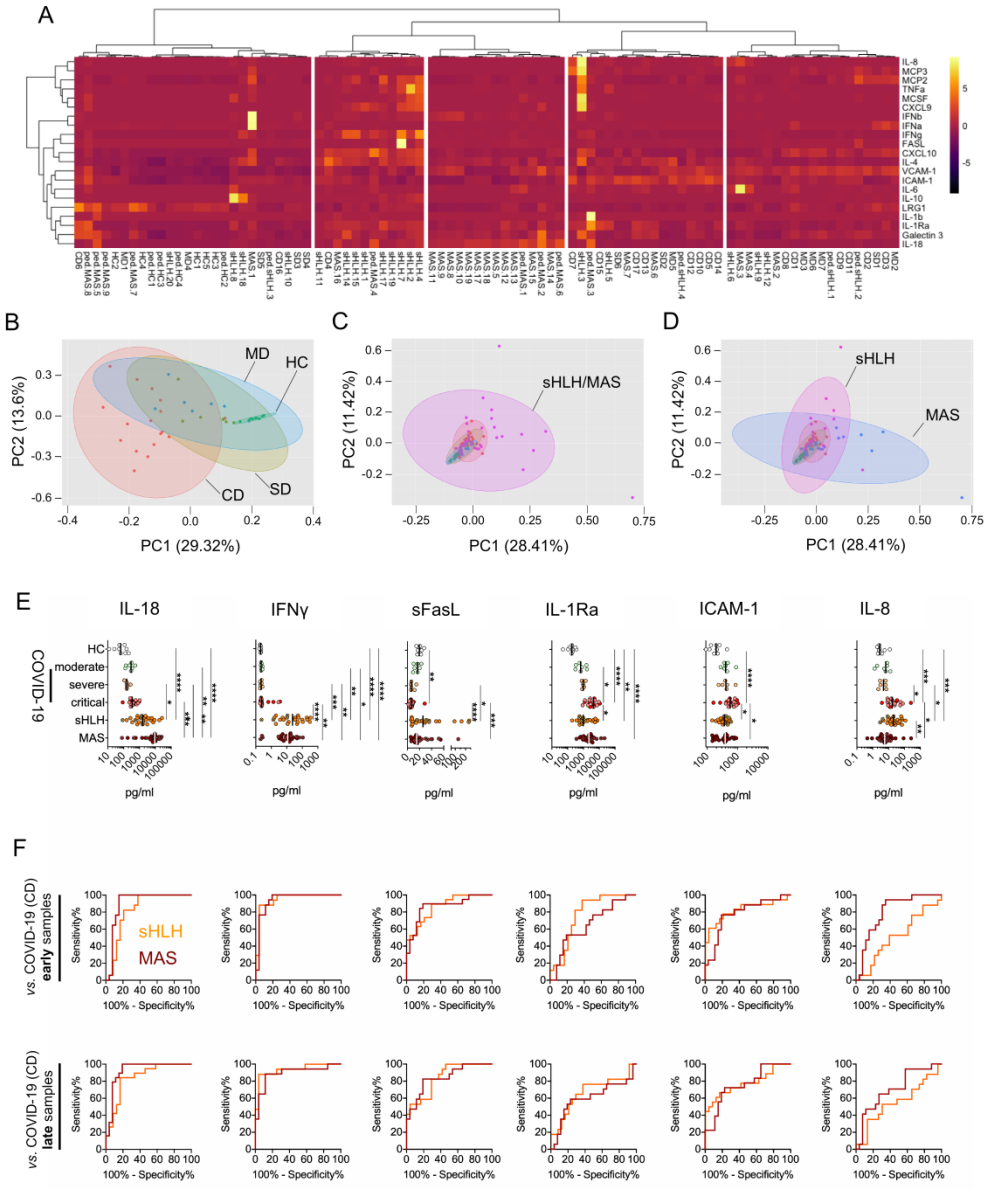
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Metastatic neoplasm | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Diabetes | 1 (3) | 1 (6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Hematologic malignancy | 3 (10) | 2 (12) | 0 (0) | 1 (14) | 0 (0) | 0 (0) |
| SAPS II, median (IQR) | | | 55 (34.5-73) | 22 (18.3-24.5) | 15 (13-28) | n.d. | n.d. |
| Leukocytes, median (IQR), $x10^9$/L | | 6.41 (4.23-8.40) | 7.51 (4.99-7.32) | 5.71 (4.89-7.32) | 4.41 (3.32-6.80) | 9.6 (2.94-13.53) | n.d. |
| Creatinine, median (IQR), mg/dL | | 0.95 (0.78-1.53) | 1.4 (0.75-1.70) | 0.8 (0.7-0.93) | 1.00 (0.80-1.00) | 1.17 (0.43-2.1) | n.d. |
| CRP, median (IQR), mg/dL | | 8.3 (3.3-16.9) | 14.2 (6.9-25.5) | 7.3 (4.3-11) | 1.6 (1.3-3.4) | 12.2 (0.8-19.1) | n.d. |
| Ferritin, median (IQR), µg/L | | 811 (582-1363) | 1084 (720-2024) | 811 (608-1426) | 596 (437-706) | 3897 (1792-10787) | n.d. |

BMI: body mass index; COPD: chronic obstructive pulmonary disease; HC: healthy control; IQR: interquartile range; MAS: macrophage activation syndrome; n.d.: not determined; SAPS: simplified acute physiology score; sHLH: secondary haemophagocytic lymphohistiocytosis

# Figure legends

**Figure 1. Serum biomarker profiles in COVID-19 and sHLH/MAS.** (**A**) Heatmap of unsupervised clustering using correlation distance and ward.D linkage of biomarker levels in the 1st serum sample following hospitalization in critical disease (CD: ARDS; n=17), severe disease (SD: no ARDS but $O_2$ supplementation; n=6) and moderate disease (MD: no ARDS, no $O_2$ supplementation, n=7) in relation to measurements of serum biomarker levels in active sHLH (adult, n=18; pediatric, n=4) and MAS (AOSD, n=17; SLE, n=2; sJIA, n=8; jSLE, n=1) patients as well as healthy controls (HC, n=9). Color coding indicates row z-score. (**B-D**) Principal component analyses (PCA) of samples as in A, analyzing (**B**) only COVID-19 samples according to disease severity or (**C, D**) comparing COVID-19 to sHLH and MAS. (**B-D**) COVID-19 disease severities are color-coded identically. (**E**) Individual biomarkers with differential expression in sHLH and MAS compared to COVID-19 are shown as scatter plots + medians (black or white). Data were analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test. (**F**) Receiver operating characteristic (ROC) curve analyses of indicated serum biomarkers differentiating critical disease (CD) COVID-19 patients' samples collected early (1st serum sample following hospitalization, upper panels) or late in disease progression (lower panels) from sHLH (orange line) or MAS (dark red line). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

**Figure 2. Dysregulation of the IL-18-IFNγ axis in classic cytokine storm syndromes compared to COVID-19. (A**, **B)** Hierarchical clustering of multiple spearman rank correlation analyses of biomarker levels in active sHLH/MAS (n=50, **A**) and critical disease (CD: n=17, **B**) COVID-19 patients. Positive associations are depicted in red, negative associations in blue. (**C**, **E**) Multiple spearman rank correlation analyses of indicated serum biomarker or cell levels in critical COVID-19 (CD: n=17, **C**) and sHLH/MAS (n=50, **E**). (**D**, **F**) In individual correlation analyses samples of deceased CD COVID-19 patients (n=7) are indicated as red diamonds (**D**). Among sHLH/MAS samples, sHLH patients (n=22) are colored in orange, MAS patients (n=28) are colored in dark red. Pediatric/adolescent sHLH (n=4) and MAS patients (n=9) are indicated as squares.

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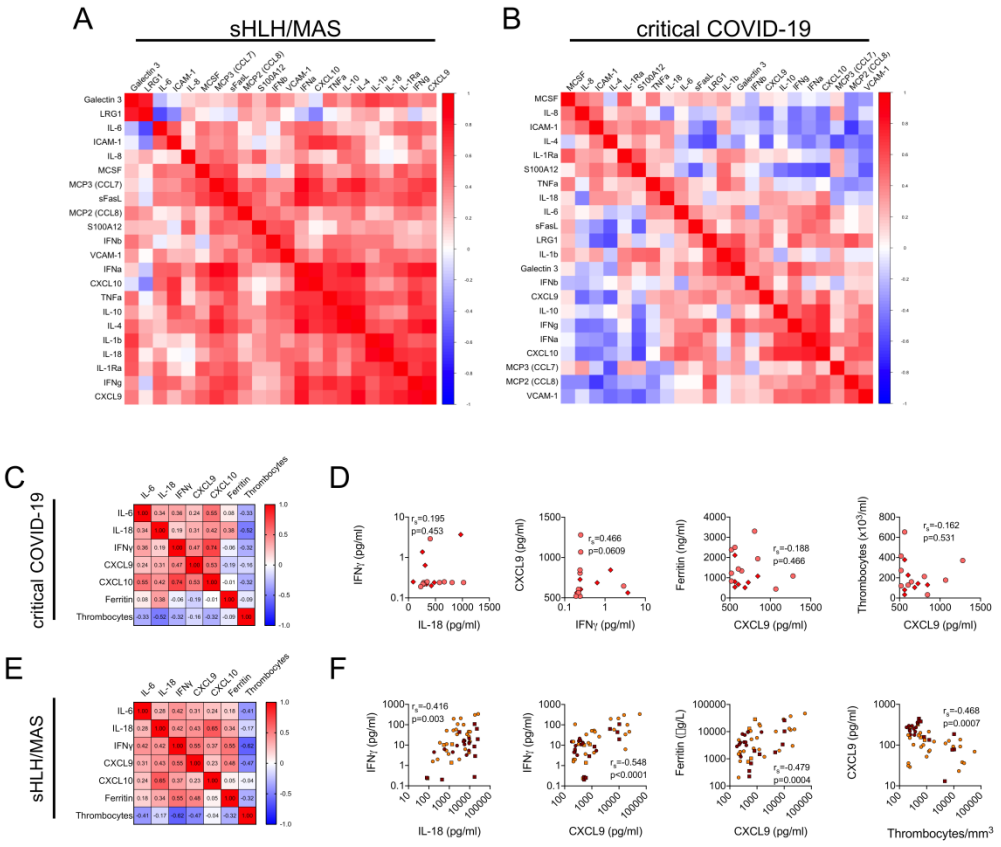


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